DIAGNOSTIC TEST FOR ALZHEIMER'S DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is based upon and claims the priority of U.S. Provisional Patent Application Serial No. 60/195,231 filed April 7, 2000 which is incorporated herein by reference.

TECHNICAL FIELD

The present invention is concerned with a diagnostic test for Alzheimer's disease.

BACKGROUND ART

Alzheimer's disease (AD) is a common progressive dementia involving loss of memory and higher cognitive function. The disease is characterized by the presence of amyloid deposits in the brains of sufferers. These deposits are found both extracellularly (amyloid plaques) and intracellularly (neurofibrillary tangles). The principal constituent of amyloid plaques is the amyloid protein (Aß) which is produced by proteolytic cleavage for the amyloid protein precursor (APP) (Evin *et al., 1994).* The principal constituent of neurofibrillary tangles is the cytoskeletal protein tau (Kosik, 1992).

One of the characteristic neurochemical changes observed in AD is the loss of acetylcholinesterase (AChE) and choline acetyltransferase activity in regions of the brain such as the cortex, hippocampus, amygdala and nucleus basalis (Whitehouse *et al.*, 1981, 1982; Struble *et al.*, 1982; Mesulam and Geula, 1988). The loss of cholinergic structure and markers correlates with the number of plaque and tangle lesions present, as well as with the clinical severity of the disease (Perry *et al.*, 1978; Wilcock *et al.*, 1982; Neary *et al.*, 1986; Perry, 1986). This loss of AChE is accompanied by an increase in butyrylcholinesterase (BuChE) (Atack et al., 1986)

Accurate diagnosis of AD during life is essential. However, clinical evaluation is at best only about 80% accurate. Therefore, there is a need to identify specific biochemical markers of AD. So far, analysis of blood or cerebrospinal fluid (CSF) has not yielded a biochemical marker of sufficient diagnostic value (Blass *et al., 1998),* although detectable differences are reported in the levels of certain proteins (Motter *et al.*, 1995).

The assay of levels of AChE and BuChE activity in the blood and the cerebrospinal fluid (CSF) have been proposed as an ante mortem diagnostic test for AD. However, no consensus has been reached as to whether the levels of AChE and BuChE are consistently affected in these tissues. The level of serum or plasma AChE has been reported to be increased (Perry *et al.*, 1982; Atack *et al.*, 1985), decreased (Nakano *et al.*, 1986; Yamamoto *et al.*, 1990) or unchanged (St. Clair *et al.*, 1986; Sirvio *et al.*, 1989) in AD patients. The level of erythrocyte AChE has been reported as either unaffected (Atack *et al.*, 1985; Perry *et al.*, 1982) or decreased Chipperfield *et al.*, 1981). The level of AChE activity in the CSF of AD patients has been reported to be decreased (most recently by Appleyard and McDonald, 1992; Shen *et al.*, 1993) or unchanged (most recently by Appleyard *et al.*, 1987; Ruberg *et al.*, 1987).

AChE and BuChE have been shown to exist as up to six different molecular isoforms, three of which are the monomeric (G1), dimeric (G2) and tetrameric (G4) isoforms (Massoulié *et al.*, 1993). The relative proportion of the different isoforms of AChE and BuChE are markedly affected in AD, with a decrease in the G4 isoform of AChE in the parietal cortex (Atack *et al.*, 1983), and an increase in the G1 isoform of

AChE (Arendt *et al.*, 1992). Similar changes have been identified in other AD brain regions including Brodman areas 9, 10, 11, 21 and 40, as well as the amygdala (Fishman *et al.*, 1986). Asymmetric collagen-tailed isoforms (A12) are increased by up to 400% in Brodman area 21, although they represent only a trace amount of the total AChE in the human brain (Younkin *et al.*, 1986).

However, to date changes in AChE and BuChE expression and isoform distribution have not been found to be of sufficient sensitivity or specificity to be useful diagnostic markers of AD.

DISCLOSURE OF THE INVENTION

There remains a need for a diagnostic test for AD based on a biochemical analysis of body fluids such as blood or CSF. The present invention provides such a test on the basis that the butyrylcholinesterase (BuChE) of AD patients shows a different glycosylation pattern to the BuChE of non-AD groups.

According to a first aspect of the present invention there is provided a method for the diagnosis of Alzheimer's disease (AD) in a patient, comprising the steps of:

(1) providing a sample of an appropriate body fluid from said patient, and

(2) detecting the presence of BuChE with an altered glycosylation pattern in said sample.

In one embodiment of the invention the relative proportion of BuChE with a specific glycosylation pattern to the total BuChE is measured.

Measurement of the relative proportion of the isoforms of BuChE with a specific glycosylation pattern to the total BuChE may be carried out in any convenient manner, for example, by using biochemical analysis techniques such as HPLC and mass spectrometry, or immunological techniques such as ELISA or, assays. However, a particularly preferred means of measuring the relative proportions of the isoforms of BuChE involves a lectin-binding analysis.

It has been established that on average approximately 93.6% of the BuChE in the CSF of AD patients binds to Concanavalin (Con A). Accordingly, in a particularly preferred embodiment of the invention, in order to detect the presence of BuChE with a specific glycosylation pattern in the sample, the binding of BuChE to Con A is determined.The percentage of BuChE bound to Con A is characteristic of the proportion of BuChE with the specific glycosylation pattern.

Also, it is particularly useful to measure the activity of unbound BuChE in each experiment, by determining the amount of BuChE unbound to Con A relative to the total BuChE in the sample.

In another embodiment of the present invention, it is particularly advantageous to compare the ratio of AChE that binds to Con A with the AChE that binds to wheat germ agglutinin (WGA), hereinafter referred to as the C/W ratio, versus the percentage of BuChE unbound to Con A. The ratio is characteristic of the different glycosylation patterns of AChE. By plotting the C/W ratio versus the percentage BuChE unbound to

Con A, the separation of patients diagnosed with AD as compared with non-AD becomes evident when viewing such a plot.

Approximately 75-95% of the AChE in the CSF of AD patients bind to Concanavalin (Con A) or wheat germ agglutinin (WGA) but with different specificity to each. For patients with AD, the C/W ratio is typically above 0. 95 and the percentage of BuChE unbound to Con A relative to the total BuChE is at least about eight percent (8%).

In an alternative embodiment of the invention there is provided a monoclonal antibody specific for BuChE with an altered glycosylation pattern used to detect its presence.

The body fluid analysed can be cerebrospinal fluid (CSF), blood or blood plasma. Advantageously, when said body fluid is blood, blood plasma is prepared from the blood for analysis.

According to a further aspect of the present invention there is provided an abnormal isoform of BuChE with an altered pattern of glycosylation and characterised in that it has a relatively lesser affinity for Concanavalin (Con A) than BuChE with an unaltered glycosylation pattern.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the correlation between the level of AChE and BChE in cerebrospinal fluid. (Black squares = controls. Open circles = AD.) This figure shows that there is a positive correlation in the levels of AChE and BChE suggesting that similar biochemical mechanisms may underly the decrease in activity of both enzymes in AD CSF.

Figure 2 is a plot of the AChE C/W ratio vs. the percentage (%) BChE unbound to Con. A. This figure shows that there is no clear correlation between these analytes. This figure also shows that by combining both measures, almost complete separation can be achieved between the AD and control groups.

Figure 3 is a plot of the percentage (%) BChE unbound to Con A vs. age (yr) for both AD and controls. This figures shows that there is no relationship between BChE glycosylation and age. Thus disease status is the only correlate.

Figure 4 is a three dimensional plot of the total BChE, C/W ratio and the percentage (%) BChE unbound to Con A showing complete separation of the AD and control groups.

BEST MODE FOR CARRYING OUT THE INVENTION

AChE, acetylcholinesterase; butyrylcholinesterase (BuChE) ChE, cholinesterase; Aβ, amylid β protein; AD, Alzheimer's disease; DP, diffuse plaques; ND, other neurological diseases; PMI, *post mortem* interval; PBS, phosphate-saline buffer; TB, Tris buffer; TSB, Tris-saline buffer; SS, salt-soluble supernatant; TS, Triton X-100-soluble supernatant; AF, amphiphilic fraction; HF, hydrophilic fraction; G^a, globular amphiphilic isoform; G^{na}, globular non-amphiphilic isoform; and agglutinins from *Canavalia ensiformis* (Concanavalin A), Con A; *Triticum vulgaris* (wheat germ), WGA; *Ricinus communis, RCA_{120}; Lens culinaris, LCA; Dolichus biflorus,* DBA; *Ulex europaeus, UEA_I; Glycine max,* SBA; *and Arachis hypogaea,* PNA.

Immobilised lectins (Con A- and LCA-Sepharose, WGA-, RCA_{120}-, DBA-, UEA_I-; SBA and PNA-agarose), phenylagarose, bovine liver catalase, *E. coli* alkaline phosphatase, polyoxyethylene-10-oleyl ether (Brij 97), Triton X-100, tetraisopropyl pyrophosphoramide (*iso*-OMPA), 1,5-bis(4-allydimethyl-ammoniumphenyl)-pentan-3-1 dibromide (BW284c51), acetylthiocholine iodide and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) were all obtained from Sigma-Aldrich Pty. Ltd. (Seven Hills, NSW, Australia). Sepharose CL-4B was purchased from Pharmacia Biotech AB (Uppsala, Sweden).

Table 1 Binding of CSF BChE to Various Lectins

	% BChE Unbound to Lectin			
	Controls	AD	DNAT	OND
Lectin				
Con A	3.0 ± 0.6	6.4 ± 0.7*	2.0 ± 0.7	2.4 ± 0.9
LCA	57.4 ± 6.2	29.4 ± 2.2	41.0 ± 1.5	39.6 ± 3.1
WGA	10.4 ± 3.7	6.2 ± 0.8	4.6 ± 0.9	4.4 ± 1.0
RCA	39.7 ± 4.0	38.5 ± 2.7	32.0 ± 9.4	42.3 ± 3.7
DBA	98.0 ± 1.2	93.8 ± 1.9	100.0 ± 0.0	83.8 ±10.5
PNA	86.6 ± 6.1	93.3 ± 1.5	92.7 ± 1.5	95.8 ± 4.3
SBA	83.7 ± 2.1	86.9 ± 1.5	96.0 ± 1.0	91.0 ± 2.4
UEA_I	92.1 ± 1.6	90.4 ± 1.7	91.3 ± 0.3	93.0 ± 3.0

* = significantly different from controls (P = 0.002).

The following Examples relate to experiments conducted with acetylcholinesterase (AchE). One skilled in the art would readily be able extrapolate from the following Examples to make a diagnostic test for Alzheimer's Disease comprising the detection of butyrylcholinesterase (BchE) with an altered glycosylation pattern

EXAMPLE 1 Lectin binding AChE experiments in AD patients.

Lumbar or ventricular CSF was obtained post mortem; 18 controls with no clinical or pathological dementia and no clinical or pathological dementia and no evidence of brain pathology, 27 cases of AD, 7 cases of dementia non-AD type (DNAT, 5 frontal lobe dementia, 1 Lewy body dementia/Parkinson's disease and 1 multi-infarct dementia/congophilic amyloid angiolpathy), and 6 cases of other neurological disorders (ND, 4 Huntington's disease, 1 schizophrenia and 1 corticobasal degeneration). The average age in the control group was 68 ± 4 years, there were 10 females and 8 males and the PMI was 40 ± 6. In the AD group the age was 81 ± 2 years, there were 13 female and 14 males and the PMI was 35 ± 6. In the ND group the age was 65 ± 6, there were 3 females and 3 males and the PMI was 45 ± 12. In the DNAT group the age was 76 ± 3, there were 4 female and 3 males and the PMI was 34 ± 11. Samples of CSF were stored at -70°C and centrifuged at 1,000 xg for 15 min prior to analysis. AChE activity was assayed at 22°C by a modified microassay of the Ellman method (Ellman *et al.* 1961). Aliquots (0. 3 ml) were mixed with 0.1 ml of Sepharose 4B in PBS (control), Concanavalin A (Con A) or wheat germ agglutinin (WGA, *Triticum vulgaris)* immobilised on Sepharose. The enzyme-lectin mixture was incubated overnight at 4°C, and then centrifuged (1,000 xg, 15 min). AChE activity was assayed in the supernatant fractions. Data were analysed using a Student's t-test.

The total AChE values in ventricular CSF samples of subjects =>60 yrs old were significantly lower in the AD group (6. 98±0. 82 nmol/min/ml) than in controls

(17. 24±4. 28 nmol/min/ml; P <0. 001). However, as reported previously, (Appleyard *et al.*,1983), the large overlap (40%) between the data prevents the use of total AChE as a significant diagnostic marker.

However, lectin-binding analysis revealed a significant difference between the AD group and controls. Approximately 75-95% of the AChE in the CSFs bound to Con A or WGA. A ratio (C/W ratio) was defined as AChE unbound to Con A divided by AChE unbound to WGA. The mean C/W ratio for the AD group was significantly different from controls (Figure 1). Of the 27 CSFs from confirmed AD, 21 samples had a C/W ratio >0. 95. All 18 control samples had C/W <0. 95, without significant differences between younger (n=5, C/W=0. 37 ± 0.10) and older subjects (n=6, 0. 38 ± 0.08) samples. No correlation in C/W ratio was noted with post mortem interval (PMI). The data are represented graphically in Figure 1.

The data indicate that lectin-binding analysis of CSF AChE could provide a diagnostic test for AD which is 80% sensitive and 97% specific. Thus it was proposed that differences observed in the glycosylation pattern of AChE in CSF may be useful as an ante mortem diagnostic marker for AD, particularly when used in combination with measurement of other biochemical markers.

EXAMPLE 2 Human brain and CSF samples for AChE Experiements

Ventricular and lumbar CSF, frontal cortical and cerebellar samples were obtained *post mortem* and stored at -80°C. Three non-AD groups of samples were defined, 1) controls with no clinical or pathological features of dementia (n = 18), 2)

individuals who showed no clinical signs of dementia but who were found to have a moderate number of non-neuritic Ab-immunoreactive diffuse plaques (DP), but no evidence of neocortical neurofibrillary changes (n = 6), and 3) individuals with various neurological diseases (ND) containing 7 cases of non-AD type dementia (5 frontal lobe dementia, 1 Lewy body dementia and 1 vascular dementia) and 7 cases of other neurological disorders (4 Huntington's disease, 1 Parkinson's disease, 1 schizophrenia and 1 corticobasal degeneration). Cases of AD were selected on the basis of their clinical history of dementia and neuropathological CERAD diagnosis (Mirra *et al.*, 1994). All the CSF samples included in the AD and ND groups were ventricular and only 5 control and 1 DP CSF samples (from a total of 18 and 6 subjects, respectively) were taken by lumbar puncture. Immunohistochemical examination of the cerebellar samples showed that, unlike the frontal cortex, none of the AD tissue possessed compact neuritic amyloid plaque deposition (data not shown), consistent with previous studies (Mann *et al.*, 1996).

It has been shown (Grass) *et al.*, 1982; Fishman *et al.*, 1986; Sáez-Valero *et al.*, 1993) that for a p*ost mortem* interval (PMI) greater than 72 hr, storage at -20°C or repeated cycles of freeze-thawing caused degradation of AChE, which confounded glycosylation analysis. Therefore, only samples with a PMI of less than 72 hr (PMI = 36 ± 4 hr) were used. There was no significant difference in PMI between each group of samples.

Preparation of samples and extraction of AChE

Samples of CSF were thawed slowly at 4°C and then centrifuged at 1,000xg for 15 min prior to use. Small pieces (0.5g) of frontal cortex and cerebellum were thawed slowly at 4°C, weighed and homogenised (10% w/v) in ice-cold Tris-saline buffer (TSB; 50 mM Tris-HCl, 1 M NaCl, and 50 mM $MgCl_2$, pH 7. 4) containing a cocktail of proteinase inhibitors (Silman *et al.*, 1978). Tissues were homogenised with a glass/Teflon homogeniser and then sonicated with 10 - 15 bursts at 50% intermittency at setting 4 using a Branson sonifier. The suspension was centrifuged at 100,000xg at 4° C in a Beckman L8-80M ultracentrifuge using a 70.1 Ti rotor for 1 hr to recover a salt-soluble ChE fraction (SS). The pellet was re-extracted with an equal volume of TSB containing 1% (w/v) Triton X-100, and the suspension centrifuged at 100,000xg at 4°C for 1 hr to obtain a Triton X-100-soluble ChE fraction (TS). This double-extraction method recovered 80-90% of the total ChE activity (SáezValero *et al.*, 1993; Moral-Naranjo *et al.*, 1996).

AChE assay and protein determination

AChE activity was determined by a modified microassay method of Ellman (Sáez-Valero *et al.*, 1993). One unit of AChE activity was defined as the number of nmoles of acetylthiocholine hydrolysed per min at 22°C. Protein concentrations were determined using the bicinchoninic acid method with bovine serum albumin as standard (Smith *et al.*, 1985).

Hydrophobic interaction chromatography on phenyl-agarose

Amphiphilic AChE forms were separated from hydrophilic forms by hydrophobic interaction chromatography on phenyl-agarose as previously described (Sáez-Valero et al., 1993). CSF (10 ml-pooled from four samples obtained from four different subjects) was applied to a column (10x1 cm) of phenyl-agarose. A hydrophilic fraction (HF) containing hydrophilic isoforms of AChE was eluted with 30 ml of TSB, and then an amphiphilic fraction (AF) containing bound amphiphilic isoforms was eluted with 50 mM Tris-HC1 (TB, pH 7.4) containing 2% (w/v) Triton X-100. Peak fractions with high AChE activity were pooled and concentrated using Ultrafree-4 Centrifugal Filter Device Biomax 10 kDa concentrators (Millipore Corporation, Bedford, MA, USA).

Sedimentation analysis

Molecular isoforms of AChE were analysed by ultracentrifugation at 150,000xg in a continuous sucrose gradient (5-20% w/v) for 18 hr at 4°C in a Beckman SW40 rotor. The gradients contained 10 ml of 50 mM Tris-HC1 (pH 7. 4) containing 0.5 M NaCl, 50 mM $MgCl_2$ and 0.5% (w/v) Brij 97. Approximately 40 fractions were collected from the bottom of each tube. Enzymes of known sedimentation coefficient, bovine liver catalase (11. 4S, $S_{20,w}$ Svedberg Units) and *E. coli* alkaline phosphatase (6.1S) were used in the gradients to determine the approximate sedimentation coefficients of AChE isoforms. A ratio of AChE species $G_4/(G_2+G_1)$, that reflected the proportion of G_4 molecules ($G_4^{na}+G_4^{a}$) versus both light globular AChE isoforms, G_2^{a} and G_1^{a} was defined. Estimation of the relative proportions of each molecular form of AChE was performed by adding the activities under each peak (G_4 or G_2+G_1) and calculating the relative percentages (recovery >95%).

Lectin-binding analysis of AChE

Samples (0.3 ml) were added to 0.1 ml (hydrated volume) of Sepharose 4B (control), Con A, WGA, RCA_{120}, LCA, DBA, UEA_I, SBA or PNA immobilised in agarose or Sepharose. The enzyme-lectin mixture was incubated overnight at 4°C with gentle mixing. Bound and free AChE were separated by centrifugation at 1000xg for 15 min at 4°C in a Beckman J2-21M/E centrifuge using a JA-20 rotor, and the unbound AChE was assayed in the supernatant fraction. Percentage of unbound AChE in the lectin incubation was calculated as (AChE unbound to lectin / AChE unbound to Sepharose) X 100. The C/W ratio was calculated according to the formula, AChE activity unbound in the Con A incubation divided by the AChE activity unbound in the WGA incubation. It was observed that this ratio detects a specific alteration in AChE glycosylation that occurs in AD CSF.

Lectin binding of CSF AChE

To examine the glycosylation of AChE, CSF samples from 18 controls and 30 cases of AD were incubated with different immobilised lectins, which recognise different sugars. AChE bound strongly to Con A, WGA and LCA but weakly to RCA_{120}, PNA, DBA, UEA_I and SBA (Table 1), suggesting that most of the enzyme was devoid of terminal galactose, terminal N-acetyl-galactosamine or fucose.

There was a small but significant difference in the binding of AChE to Con A and WGA between the AD group and controls (Table 1). As the percentage of AChE unbound in the AD CSF was increased for Con A and decreased for WGA, a ratio (C/W

= [% AChE that does not bind to Con A] / [% AChE that does not bind to WGA]) was defined, which provided greater discrimination between the two groups (Table 1). Using this method, it was found that the mean C/W ratio for the AD group was significantly greater than for the other control groups, including cases with diffuse plaques (non-demented, DP), and patients with other neurological and neuropsychiatric diseases (ND) (Fig. 2), consistent with the results shown in Example 1. Of the 30 CSF samples from confirmed AD cases, 24 samples were above a cut-off value of C/W = 0.95 (Fig. 2). Only one sample from 18 controls, one out of 6 samples from cases with diffuse plaques, and one out of 14 samples from the other neurological diseases group, a frontal lobe dementia case, were above this value. The 6 AD samples with C/W ratios lower than 0.95 had C/W ratios > 0.60, a value higher than the C/W mean of the non-AD groups (control = 0. 53 ± 0.1; DP = 0. 46 ± 0.2; ND = 0.53 ± 0.1).

No correlation could be found between the C/W ratio and the PMI that could suggest that different C/W ratio in the AD group was due to differences in PMI. Furthermore, there was no significant difference in the PMI between the AD (33 ± 6 hr) and non-AD samples (40 ± 6 hr).

CSF samples were additionally analysed for total AChE activity (Fig. 2). As previously reported (Appleyard *et al.*, 1983; Atack *et al.* , 1988), the CSF from patients with AD had significantly lower AChE activity (6.5 ± 0.8 U/ml) than controls (15.8 ± 2.9 U/ml) or patients with other diseases (12.4 ± 2.4 U/ml). However, the C/W ratio was a more reliable index of clinical status than the total level of AChE activity in the CSF (Fig. 2).

AChE isoforms in CSF

To determine whether the alteration in glycosylation was due to changes in a specific isoform of AChE, CSF samples were analysed by hydrophobic interaction chromatography to separate amphiphilic (G^a) and hydrophilic species (G^{na}) (Fig. 3), and by sucrose density gradient centrifugation in 0.5% (w/v) Brij 97 to separate individual molecular weight isoforms (G_4,G_2 and G_1) (Fig. 3). A decrease in the proportion of G_4, AChE in AD CSF compared to controls (Fig. 4, top panels) was observed. The ratio of (G_4,/(G_2+G_1) was significantly ($P < 0.01$) higher in controls (1. 80 ± 0.12; n = 4) than in AD cases (1.16 ± 0.12; n = 4). To separate hydrophilic isoforms from amphiphilic isoforms, CSF was fractionated by hydrophobic interaction chromatography on phenyl-agarose (Fig. 3). A smaller percentage of AChE in the normal CSF bound to phenyl agarose (12 ± 3 %, n = 4) than in the AD CSF (38 ± 4%, n = 4; $P < 0.001$). Sedimentation analysis of the unbound hydrophilic fraction (HF) showed a main peak of 10.8S, consistent with a hydrophilic tetrameric (G_4^{na}) isoform (Atack *et al.*, 1987), as well as a small amount of lighter AChE isoforms, 5.1S dimers and 4.3S monomers (Fig. 4). The bound amphiphilic fraction from the phenyl-agarose column contained a minor peak of 9.0-9.5S (probably an amphiphilic tetramer, G_4^a and a major peak of amphiphilic globular dimer (G_2^a, 4.2S) and monomer (G_1^a, 3.1S). The level of the amphiphilic light isoforms was greater in the AD CSF than in controls (Fig. 4).

Glycosylation of individual AChE isoforms in CSF

Incubation of the HF and AF with immobilised Con A and WGA showed that there was an increase in the C/W ratio in AD CSF, and that the high C/W ratio was associated with an amphiphilic fraction containing dimers and monomers (Fig. 4). The data indicate that the contribution of G_2 and G_1 AChE in AD CSF was mainly responsible for the increased C/W ratio of total AChE in the AD CSF.

Levels of AChE in frontal cortex and cerebellum

To determine whether the changes in AChE glycosylation reflect a change in the expression or glycosylation of brain AChE isoforms, the levels of AChE activity in samples of frontal cortex and cerebellum were examined. Samples were homogenised with salt and Triton X-100 to extract soluble and membrane-bound AChE isoforms, and then the AChE activity determined in both fractions (Table 2). The frontal cortex samples from AD patients had significantly less AChE activity in the Triton X-100-soluble (TS) fraction (~40%), with no difference in levels in the salt-soluble (SS) fraction compared with controls (Table 3). The results are consistent with previous studies that indicate that the major G_4 isoform is decreased only in the TS fraction (Younkin *et al.*, 1986; Seik *et al.*, 1990). A small but significant decrease (~15%) in the protein content of the TS fraction of both AD and ND groups was also observed. The level of AChE in the frontal cortex samples of the ND group was significantly different from controls in both the SS and TS fraction (Table 2). However, as the ND group was heterogeneous (2 frontal lobe dementia, 1 Huntington's disease and 1 Parkinson's disease), the significance of changes in AChE levels is unclear. Levels of AChE in cerebellum were also significantly decreased in the TS fraction from the AD group (Table 2).

Glycosylation of AChE in frontal cortex and cerebellar

To determine whether different glycosylation pattern of AChE in AD CSF is also present in the AD brain, the glycosylation of brain AChE was examined by lectin binding. Homogenates from frontal cortex and cerebellum were incubated with immobilised Con A or WGA and the amount of activity unbound was calculated. In the AD frontal cortex, the % AChE activity that did not bind to Con A or WGA was significantly different from controls (Table 3). Similar to the CSF AChE, the C/W ratio of frontal cortex AChE was greater in AD than in non-AD samples (Table 3). This increase was due to a large increase in the amount of AChE that did not bind to Con A, and was in spite of an increase in the amount of AChE that did not bind to WGA (Table 3). There was no increase in the C/W ratio in the DP and ND group (Table 3). No difference in lectin binding was observed between AD and non-AD groups in the cerebellar fractions (Table 3.)

AChE isoforms in frontal cortex and cerebellum

To determine the cause of the altered glycosylation in AD brain, the pattern of AChE isoforms in the frontal cortex and cerebellum was examined. Equal volumes of SS and ST supernatants (total AChE activity) were pooled and then analysed by sucrose density gradient sedimentation with 0. 5% (w/v) Brij 97 to separate the major AChE isoforms (Fig. 5). Based on their sedimentation coefficients (Atack *et al.*, 1986; Massoulié *et al.*, 1982) it was possible to identify hydrophilic (G_4^{na}, 10.7 ± 0.1S) and amphiphilic tetramers (G_4^{a}, 8.6 ± 0.1S) amphiphilic dimers (G_2^{a}, 4.7 ± 0.1S) and

monomers (G_1^a, 3.0 ± 0.1S) of AChE (Fig. 6). There were no differences in the sedimentation coefficient (S) of individual isoforms from each group. Due to the overlap in the sedimentation coefficients between AChE G_4^{na} and G_4^a, it was not possible to separate these isoforms completely (Fig. 5). However, the contribution of G_4^a was greater than G_4^{na}. Asymmetric (A_{12}) AChE isoforms were identified in trace amounts (2-5%) in some of the fractions.

A significant decrease in G_4, (40% of the mean control value, $P < 0.001$) and in G_2+G_1 AChE (60% of the mean control value, $P = 0.002$) was detected in the fractions from AD frontal cortex. This change in the relative proportion of AChE isoforms was reflected in the $G_4/(G_2+G_1)$ ratio, which was significantly lower in the AD samples (Table 3). Interestingly, a similar and statistically significant decrease was found in the $G_4/(G_2+G_1)$ ratio for the DP subjects. This change in ratio was due to a 25% increase in the level of G_2+G_1 and a small decrease (10%) in G_4 AChE, although neither change on its own was statistically significant. No variation in AChE $G_4/(G_2+G_1)$ was found in the AD cerebellum (Table 3), despite a statistically significant decrease (40%) in AChE in the TS fraction (Table 2) and in the total level of G_4 AChE (G_4 in controls = 380 ± 40 U/ml, G_4 in ADs = 195 ± 70 U/ml; $P = 0.008$).

Glycosylation of individual AChE isoforms in frontal cortex and cerebellum

Since it was found that the ratio of AChE was altered in the frontal cortex of AD patients, steps were taken to ascertain whether the increase in the C/W ratio of brain AChE was due to a change in glycosylation or in the expression of a specific isoform of

AChE. Individual AChE isoforms were separated by sucrose gradient centrifugation and then fractions from the G_4 or G_2+G_1 peaks were pooled, dialysed against TSB-Triton X-100 buffer and concentrated by ultrafiltration. AChE isoforms were then assayed by lectin binding and a C/W ratio calculated for each isoform (Fig. 5).

No differences were observed in the C/W ratio of G_4, AChE between the AD and non-AD groups (Fig. 5). However, in all frontal cortex samples the G_2+G_1 fraction possessed C/W ratios >1.00, demonstrating that G_2 or G_1 AChE is glycosylated differently from the G_4 isoform. Moreover, the C/W ratio for G_2+G_1 AChE was higher in the AD group than controls or DP. Similarly, the C/W ratio of the amphiphilic fraction from CSF (containing predominantly G_2+G_1 AChE) was higher in the AD group than in controls (Fig. 3). There was no correlation between the G_4/ (G_2+G_1) ratio and the C/W ratio in the DP group in frontal cortex. In the cerebellum, no differences were observed in the C/W ratios of G_4 AChE or G_2+G_1 AChE between AD and non-AD groups (Fig. 4). The G_2+G_1 fractions, from both AD and non-AD cerebellar groups, had a C/W < 0.50, in contrast to the same fraction from frontal cortex (C/W > 1.00) indicating differences in the pattern of glycosylation of G_2+G_1 AChE between both brain areas.

This example shows that AChE is glycosylated differently in the frontal cortex and CSF of AD patients compared with AChE from non-AD groups including patients with non AD-type dementias. This difference in glycosylation is due to an increase in the proportion of differentially glycosylated amphiphilic dimeric and monomeric AChE in the AD samples. The results suggest that the abnormally glycosylated AChE in AD

CSF may be derived from the brain as a similar difference in glycosylation was also found in the frontal cortex of AD patients.

Table 1. Lectin-binding of AChE in CSF.

Lectin	AChE unbound (%)	
	Control	AD
Con A	5.5 ± 0.8	10.1 ± 1.1[b]
WGA	11.3 ± 1.7	7.0 ± 0.6[b]
Con A / WGA (C/W)	0.53 ± 0.1	1.37 ± 0.1[a]
LCA	17.2 ± 4.2	15.0 ± 1.3
RCA_{120}	74.1 ± 3.4	70.8 ± 2.7
SBA	83.0 ± 2.1	82.2 ± 1.9
UEA_I	91.6 ± 2.2	87.6 ± 1.9
PNA	92.4 ± 1.7	92.3 ± 1.4
DBA	98.9 ± 0.8	95.8 ± 1.7

All the CSFs were taken *post mortem* and the diagnosis confirmed by pathological examination. CSF from normal subjects (Control group: n= 18; 67±4 years at death; 11 Females / 7 Males) and AD patients (AD group: n= 30; 79±2 y; 15F/15M) were incubated either with an equal volume of the different immobilized lectins, and then centrifuged. AChE was assayed in the supernatant fractions. The data represent the means ± SEM. [a] Significantly different ($P < 0.001$) from the control group as assessed by Student's *t* test; [b] significantly different ($P < 0.05$) from the control group as assessed by Student's *t* test.

Table 2. AChE activity and protein levels in human frontal cortex and cerebellum

	AChE activity (U/ml)		Protein (mg/ml)	
Group/Source	SS	TS	SS	TS
Control				
Frontal Cortex (n=11; 63±5 y; 7F/4M)	3.7 ± 0.4	15.1 ± 1.5	2.1 ± 0.1	2.4 ± 0.1
Cerebellum (n=7; 66 ±5 y; 4F/3M	64 ± 6	264 ± 25	2.5 ± 0.1	1.9 ± 0.1
DP				
Frontal Cortex (n= 6; 81 ±2 y; 4F/2M)	5.5 ± 0.9	12.7 ± 1.7	2.1 ± 0.1	2.2 ± 0.1
Cerebellum (n= 5; 81 ±3 y; 3F/2M)	49 ± 8	182 ± 46	2.6 ±0.1	1.9 ± 0.1
ND				
Frontal Cortex (n= 4; 67 ±9 y; 2F/2M)	5.4 ± 0.6[a]	9.3 ± 1.7[b]	2.1 ± 0.2	2.0 ± 0.1[b]
Cerebellum (n= 2; 78 ±14 y; 1F/1M)	45 ± 8	160 ± 50	2.7 ± 0.2	2.3 ± 0.2
AD				
Frontal Cortex (n= 14; 73 ±3 y; 8F/6M)	3.7 ± 0.3	9.0 ± 0.9[a]	2.1 ± 0.1	2.1 ± 0.1[a]
Cerebellum (n= 7; 73 ±6y; 5F/2M)	48 ± 12	160 ± 28[b]	2.6 ± 0.1	2.0 ±0.1

Tissue from frontal cortex or cerebellum was homogenized and salt-soluble (SS) and Triton X-100-soluble (TS) extracts obtained. The extracts were then assayed for AChE and protein. DP = non-demented subjects with diffuse plaques; ND = individuals with other neurological diseases and dementias of non-AD type; AD = individuals with Alzheimer's disease. F = female; M = male; y = age in years. Values are means ± SEM. [a] Significantly different ($P < 0.005$) from the control group as assessed by Student's *t* test; [b] significantly different ($P < 0.05$) from the control group as assessed by Student's *t* test.

Table 3. Lectin binding and AChE isoforms in frontal cortex and cerebellum

Group/Source	Lectin binding		AChE ratio	
	AChE unbond to Con A(%)	AChE unbound to WGA (%)	C/W	$G_4/ (G_2+G_1)$
Control Frontal Cortex (n=11; 63±5 y; 7F/4M)	6.9 ± 0.8	12.3 ± 1.2	0.56 ± 0.03	1.90 ± 0.14
Cerebellum (n=7; 66 ±5 y; 4F/3M)	1.8 ± 0.1	10.7 ± 0.9	0.18 ± 0.02	3.02 ± 0.2
DP Frontal Cortex (n= 6; 81 ±2 y; 4F/2M)	7.4 ± 0.8	15.0 ± 1.0	0.50 ± 0.06	1.32 ± 0.12[b]
Cerebellum (n= 5; 81 ±3 y; 3F/2M)	2.9 ± 0.7	12.2 ± 1.3	0.23 ± 0.05	2.18 ± 0.33
ND Frontal Cortex (n= 4; 67 ±9 y; 2F/2M)	7.0 ± 0.6	13.2 ± 1.2	0.47 ± 0.05	2.61 ± 0.73
Cerebellum (n= 2; 78 ±14 y; 1F/1M)	1.8 ± 0.2	10.1 ± 0.3	0.21 ± 0.10	2.50 ± 0.70
AD Frontal Cortex (n= 14; 73 ±3 y; 8F/6M)	13.1 ± 1.3[a]	19.7 ± 1.4[a]	0.66± 0.03[b]	1.34 ± 0.18[b]
Cerebellum (n= 7; 73 ±6y; 5F/2M)	2.4 ± 0.3	13.5 ± 2.3	0.19 ± 0.02	2.33 ± 0.49

SS and TS fractions from frontal cortex and cerebellum were pooled in equal volumes and then analyzed by lectin binding using immobilized Con A and WGA. The C/W ratio was calculated as defined in Table 2. Aliquots of the supernatants (SS+TS) were also analyzed by sucrose density gradient sedimentation to identify AChE isoforms. Values are means ± SEM. [a] Significantly different ($P < 0.005$) from the control group as assessed by Student's *t* test;[b] significantly different ($P < 0.05$) from the control group as assessed by Student's *t* test.

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